Skin and Bones

CHAPTER 8

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Summary

In 2007, we reported two brothers with a multisystem disorder encompassing progressive mitral valve insufficiency, osteopenia, thoracic kyphosis, craniofacial dysmorphism, dermal fibrosis, and severe nodulocystic acne [1]. In these patients, we identified a novel homozygous *MMP14* c.332G>A missense mutation. This gene encodes matrix metalloproteinase 14, a membrane-bound endopeptidase that primarily cleaves structural components of the extracellular matrix (ECM), which is expressed in various tissues including bone and skin [2-5]. Multiple lines of evidence suggested that this mutation could be pathogenic. As shown in **Chapter 1**, *in silico* analysis predicted that the resulting p.R111H substitution would be damaging. Secondly, a homozygous MMP14 p.T17R mutation has previously been reported in patients with a clinical diagnosis of Winchester syndrome (WS), a constellation of features similar to our patients' phenotype [6, 7]. Thirdly, the mouse models *Sabe* and *Cartoon* with homozygous MMP14 p.R92C and p.S466P missense mutation, respectively, share many skeletal features with our patients [8, 9]. Fourthly, there is significant phenotypical overlap between our patients and individuals with Frank-Ter Haar syndrome (FTHS) and multicentric osteolysis, nodulosis, and arthropathy (MONA), which are caused by homozygous loss-of-function mutations in *SH3PXD2B* respectively *MMP2* [10-16]. Importantly, the protein products of these three genes directly cooperate in the formation of podosomes, which are specialised membrane protrusions involved in ECM remodelling and invasive cell motility [17-20]. Together, this evidence led us to diagnose our patients with Wichester syndrome, pending confirmation of the p.R111H mutation's pathogenicity. In this thesis, we aimed to elucidate how mutation of *MMP14* results in the WS phenotype.

In **Chapter 2**, we first assessed how our novel p.R111H mutation, and previously reported human and murine mutations, affect MMP14. MMP14 is synthesised as a latent pre-proenzyme, whose activity is controlled by different processes, including proteolytic activation of the zymogen and exposure at the cell surface [2, 3, 21-24]. To reach the plasma membrane, the full-length zymogen contains an amino-terminal signal peptide (SP) for its insertion into the endoplasmatic reticulum (ER) membrane during translation [2, 3]. Next to the SP lies a prodomain that keeps the adjacent catalytic domain inactive [2, 3, 21, 25-27]. After ER insertion, pro-MMP14 is activated by sequential cleavage of its N-terminus by MMPs and proprotein convertases (PCs), and is subsequently trafficked to the plasma membrane [22, 28-32]. The abovementioned mutations are all present at sites that are thought to be crucial for normal activation and/or trafficking of MMP14: the SP (p.T17R), PC recognition motifs (p.R92C and p.R111H), and the hemopexin-like (Hx) domain (p.S466P) [2, 3, 7-9, 28-30, 33-35]. To assess the effect of these mutations on MMP14 processing, trafficking, and activity, we developed a novel *in vitro* model consisting of fibroblasts expressing either wild type

(WT) or mutant MMP14 with two different tags at their amino respectively carboxyl terminus. As expected, the full-length WT fusion protein was sequentially processed along the biosynthetic-exocytotic pathway and finally localised at the cell surface as a functionally active enzyme. We provided the first direct evidence for MMP14 SP removal in the ER *in vitro*. The p.T17R mutation impaired ER insertion and subsequent processing and trafficking of MMP14. In contrast, MMP14 R111H was processed and trafficked seemingly normally. However, the p.R111H mutation partially impaired MMP14's catalytic activity and its stimulatory effect on cell migration *in vitro*, suggesting R^{111} is a crucial residue for pro-MMP14 activation. It is possible that the impaired activity is caused by a subtle aberration in pro-domain cleavage that we were unable to detect by Western blot. Compared to the patients originally reported by Winchester et al., our patients had a relatively mild phenotype, correlating best with mutant MMP14's ability to activate pro-MMP2 *in vitro* [6]. Our results thus confirmed the pathogenicity of the novel hypomorphic *MMP14* allele, causing a mitigated form of WS. Compared to WS patients, the *Mmp14* mutant mice have a more severe phenotype. In line with this, the p.R92C mutation dramatically impaired MMP14's intracellular trafficking and activity *in vitro*. This implies a more important role for the R^{89}-R-P-R-C^{93} PC recognition motif in MMP14 activation and subsequent trafficking than previously thought [23, 31, 36]. Although the *Sabe* and *Cartoon* phenotype are virtually indistinguishable and the respective mutations impaired MMP14's trafficking and pro-MMP2 cleavage, MMP14 S466P retained its ability to digest gelatin and stimulate cell motility [8, 9]. A possible explanation for this discrepancy is that in contrast to MMP14 R92C, some MMP14 S466P still makes it to the plasma membrane, leaving its functions that do not require homodimerisation intact [37-40]. Homodimerisation might be impaired by a dose effect, or by a direct effect of the p.S466P mutation on the Hx domain [41]. In addition, the p.S466P mutation might alter MMP14's substrate specificity, enhancing gelatin digestion, but not pro-MMP2 cleavage. Taken together, we demonstrated that these four mutations each impair MMP14 functional activity in a unique manner, resulting in a similar clinical phenotype. We subsequently used 3D *in vitro* and *in vivo* models to assess how the loss of MMP14 function resulted in specific aspects of the WS phenotype.

One aspect of the WS and FTHS phenotype of our particular interest is acne [1, 6, 10, 11, 42]. Although it is well known that acne is characterised by cystic sebaceous glands i.e. comedones, it is unknown why such cystic changes occur. The sebaceous gland is, in essence, a hollow, branched epithelial structure that is separated from the type I collagen-rich dermis by a basement membrane [43]. Morphogenesis of such structures generally relies on *de novo* lumen formation and branching morphogenesis [44]. Planar cell polarity (PCP) and ECM remodelling play a crucial role in these two processes and when disturbed, can result in cyst formation [45-48]. Interestingly, multiple cell types

depend on MMP14's catalytic activity for branching morphogenesis, and on cell surface localisation of MMP14 for normal PCP [49-52]. Therefore, we hypothesised that disrupted luminogenesis and branching morphogenesis of the sebaceous gland due to impaired function of MMP14 or, given their direct functional link outlined above, SH3PXD2B, could underly comedogenesis. As the acne in our patients was successfully treated with 13-is retinoic acid, we hypothesised that this drug could correct the putative defective branching morphogenesis [1]. In **Chapter 3**, we conducted a pilot study to assess the effects of *Mmp14* or *Sh3pxd2b* knockdown (KD) and retinoid treatment on luminogenesis and branching morphogenesis of epithelial cells *in vitro* [53, 54]. We demonstrated that KD of either of these genes impaired lumen formation in reconstituted basement membrane (Matrigel™) and reduced branching morphogenesis in type I collagen gel, whereas retinoid treatment directly stimulated both processes. The disturbed luminogenesis upon KD of *Mmp14* or *Sh3pxd2b* is suggestive of impaired PCP [52]. In addition, the observed shortened tubule length in crosslinked type I collagen matrix suggests that KD of *Mmp14* or *Sh3pxd2b* impairs ECM degradation [55, 56]. Finally, the reduced organoid size in type I collagen gel upon KD could additionally be caused by a reduction in cell proliferation. The latter two mechanisms are likely linked, as IMCD cells isolated from *Mmp14* KO mice were previously reported to have a proliferative defect that depended on MMP14's collagenolytic activity [57]. The fact that KD of either *Mmp14* or *Sh3pxd2b* caused a similar effect *in vitro* suggests that a shared pathway is affected. Given its role in ECM remodelling, podosome function is the most likely candidate. Retinoids could act through stimulating podosome formation, as we demonstrated that 13-cis retinoic acid stimulated trafficking of MMP14 S466P to podosomes *in vitro*. This could explain the therapeutic effect of 13-cis retinoic acid in acne treatment, although additional tissue-specific effects are likely involved [58, 59]. Future studies should repeat our experiments with sebocytes to assess whether these processes could affect the sebaceous gland. Such sebocyte 3D model could potentially be used in screening for anti-acne therapeutics.

Apart from acne, the WS phenotype is characterised by craniofacial dysmorphism and generalised osteopenia [1, 6]. The majority of the affected craniofacial skeletal elements are of neural crest (NC) origin, the primary embryonic structure contributing to the development of the face [60-62]. After their induction during embryogenesis, NC cells delaminate and migrate extensively, for which ECM remodelling is essential [60, 61, 63-65]. As MMP14 is involved in ECM remodelling and invasive cell motility, we hypothesised that impaired cranial NC migration underlies the WS craniofacial phenotype [4, 5, 21, 66]. To test this hypothesis, and to study the underlying cause of osteopenia in WS, we decided to use a zebrafish model. Zebrafish form the same skeletal tissues as humans, which furthermore develop in a similar manner. This especially holds true for the zebrafish skull bones, the majority of which is of NC origin [67-70].

Zebrafish have two well-conserved MMP14 paralogs, Mmp14a and Mmp14b, which are expressed in the head mesenchyme during development [71-76]. The rapid external development and optical transparency of zebrafish embryos furthermore enable live *in vivo* imaging of NC cells [77-79]. In **Chapter 4**, we used the CRISPR/Cas9 approach to successfully knock out (KO) both *mmp14a* and *mmp14b* in zebrafish. Our *mmp14a/b* KO fish recapitulated essential aspects of the WS phenotype, including stunted growth, gradually worsening craniofacial anomalies, hyperkyphosis, osteopenia (albeit limited to the skull), and a shortened lifespan [1, 6]. In contrast to our hypothesis, NC induction, delamination, pharyngeal arch invasion, and differentiation into larval craniofacial cartilage elements were unaffected by *mmp14a/b* KO. Previously described craniofacial defects in *mmp14a* of *mmp14b* morphants are likely off-target effects of the used Morpholino oligonucleotides [74, 75, 80]. In our *mmp14a/b* KO larvae, skeletal mineralisation during larval-to-juvenile metamorphosis was unaffected [81]. Adult mutant fish showed impaired endochondral/perichondral and intramembranous ossifying bones of both NC and mesodermal origin, further arguing against an NC-specific problem [68, 82]. Affected bones generally contained relatively little amounts of bone matrix with altered collagen content, clusters of multinucleated cells, and a relatively volumous, disorganised cartilage core. In mice, loss of MMP14 impairs timely cartilage removal during both ossification types and is accompanied by excessive absorption of mineralised bone matrix, resulting in similar bone abnormalities as those observed in our *mmp14a/b* KO fish [51, 83]. Although it was previously demonstrated *in vitro* that MMP14 stimulated osteoblast differentiation and inhibited osteoclast differentiation and activation, additional assays should shed light on the exact processes and cell types involved in the observed skeletal phenotype in the *mmp14a/b* KO fish [84-86]. In addition, it is still unknown to what extent impaired podosome function is involved in the resulting human, murine and zebrafish phenotype. The generation of various combinations of *mmp14a/b* KO, *sh3pxd2b* KO and *mmp2* KO zebrafish might shed light on how their protein products interact, at least in fish, in bone remodelling. Finally, the newly generated *mmp14a/b* KO fish could be a suitable model for development of novel therapeutics addressing not only the WS bone phenotype, but also low bone density in general.

Samenvatting

In 2007 beschreven wij twee broers met een multisysteemaandoening, bestaande uit progressieve mitraalklepinsufficiëntie, osteopenie, thoracale hyperkyfose, craniofaciale dysmorfie, dermale fibrose, en ernstig nodulocysteus acne [1]. Recent toonden wij aan dat deze patiënten homozygoot zijn voor een niet eerder gerapporteerde c.332G>A missense mutatie in *MMP14*. Dit gen codeert voor matrix metalloproteinase 14, een membraangebonden endopeptidase dat vooral structurele componenten van de extracellulaire matrix (ECM) hydrolyseert en tot expressie komt in diverse weefsels, waaronder de huid en bot [2-5]. Het is om meerdere redenen aannemelijk dat deze mutatie pathogeen is. Zoals getoond in **Hoofdstuk 1**, voorspelde *in silico* analyse dat de resulterende p.R111H substitutie schadelijk is. Eerder werd een homozygote MMP14 p.T17R mutatie geïdentificeerd in twee patiënten met Winchester syndroom (WS), die qua fenotype sterk lijken op onze patiënten [6, 7]. Ten derde hebben de muismodellen *Sabe* en *Cartoon*, met respectievelijk homozygote MMP14 p.R92C en p.S466P mutatie, skeletafwijkingen vergelijkbaar met die van onze patiënten [8, 9]. Ten vierde is er een sterke gelijkenis tussen het fenotype van onze patiënten en dat van mensen met Frank-Ter Haar syndroom (FTHS) dan wel multicentrische osteolyse, nodulose, en arthropathie (MONA). FTHS en MONA worden veroorzaakt door homozygote mutaties in respectievelijk *SH3PXD2B* en *MMP2* [10-16]. Deze twee genen coderen voor eiwitten die direct samenwerken met MMP14 in de vorming van podosomen, gespecialiseerde membraanuitstulpingen die betrokken zijn bij ECM remodellering en invasieve celmotiliteit [17-20]. Om deze vier redenen hebben wij onze patiënten gediagnosticeerd met WS, hoewel de pathogeniciteit van de MMP14 p.R111H mutatie nog bevestogd moest worden. In dit proefschrift probeerden wij te ontrafelen hoe mutatie van *MMP14* leidt tot het WS phenotype.

In **Hoofdstuk 2** hebben wij onderzocht of onze nieuw ontdekte p.R111H mutatie daadwerkelijk pathogen is, en hoe deze en eerder gerapporteerde mutaties MMP14 beïnvloeden. MMP14 wordt gesynthetiseerd als een latent pre-proenzym, waarvan de activiteit gereguleerd wordt door verschillende processen waaronder proteolytische activatie van het zymogeen en blootstelling op het celoppervlak [2, 3, 21-24]. Om het plasmamembraan te bereiken bevat pro-MMP14 een signaalpeptide (SP) aan zijn aminoterminus voor insertie in het endoplasmatisch reticulum (ER) membraan tijdens translatie [2, 3]. Naast het SP ligt een prodomein, dat het nabij gelegen katalytisch domein inactief houdt [2, 3, 21, 25-27]. Na ER insertie wordt pro-MMP14 geactiveerd door sequentiële hydrolyse van zijn aminoterminus door MMPs en proproteine convertasen (PCs) en wordt actief MMP14 vervolgens naar het plasmamembraan getransporteerd [22, 28-32]. Al de bovengenoemde mutaties zijn aanwezig op plekken die cruciaal worden geacht voor activatie en/of transport van MMP14: het SP (p.T17R), de PC herkenningsmotieven (p.R92C en p.R111H), en het hemopexine (Hx) domein

(p.S466P) [2, 3, 7-9, 28-30, 33-35]. Wij hebben het effect van deze mutaties op hydrolyse, transport en activiteit van MMP14 onderzocht aan de hand van een nieuw ontwikkeld *in vitro* model. Dit model bestond uit fibroblasten die wild-type (WT) dan wel mutant MMP14 met twee verschillende labels aan respectievelijk hun amino- en carboxylterminus tot expressie brachten. Zoals verwacht, werd het WT fusie-eiwit sequentieel gehydrolyseerd in de biosynthetische-exocytaire route en bevond zich uiteindelijk op het celoppervlak als functioneel actief enzym. Dit leverde tevens het eerste directe bewijs voor verwijdering van het SP van MMP14 in het ER *in vitro*. De p.T17R mutatie verstoorde ER insertie en daaropvolgende hydrolyse en transport van MMP14. Daarentegen verliep de hydrolyse en het transport van MMP14 R111H schijnbaar normaal. De p.R111H mutatie verstoorde de katalytische activiteit en het stimulerende effect op celmigratie van MMP14 *in vitro* echter deels, wat suggereert dat R^{111} een cruciaal residu is voor MMP14 activatie. Het is mogelijk dat de verminderde activiteit veroorzaakt wordt door een subtiele afwijking in prodomeinhydrolyse, die wij niet konden detectered door middel van Western blot. Vergeleken met de door Winchester et al. beschreven patiënten, hadden onze patiënten een relatief mild fenotype; dit correleerde het beste met de mate van pro-MMP2 activatie door mutant MMP14 *in vitro* [6]. Onze resultaten bevestigden de pathogeniciteit van een nieuw, hypomorf *MMP14* allel, dat een milde vorm van WS veroorzaakt. Vergeleken met de WS patiënten, hebben de *Mmp14* mutante muizen een ernstiger fenotype. In overeenstemming hiermee verstoorde de p.R92C mutatie het intracellulair transport en de activiteit van MMP14 *in vitro* dramatisch. Dit impliceert dat het R^{89}-R-P-R-C^{93} PC herkenningsmotief een belangrijkere rol speelt in deze processen dan eerder werd aangenomen [23, 31, 36]. Hoewel het *Sabe* en *Cartoon* phenotype nagenoeg identiek zijn aan elkaar en de respectievelijke mutaties de pro-MMP2 activatie door MMP14 ernstig verstoorden, behield MMP14 S466P zijn vermogen tot hydrolyse van gelatine en stimulatie van celmotiliteit *in vitro* [8, 9]. Een mogelijke verklaring voor deze discrepantie is dat in tegenstelling tot MMP14 R92C, enig MMP14 S466P het celmembraan bereikt, en daarmee functies die onafhankelijk zijn van homodimerisatie gespaard blijven [37-40]. Homodimerisatie kan verstoord zijn door een dosis-effect, of door een direct effect van de p.S466P mutatie op het Hx domein [41]. Daarnaast zou de p.S466P mutatie de substraatspecificiteit van MMP14 kunnen veranderen, wat de proteolyse van gelatine, maar niet van pro-MMP2, vergemakkelijkt. Samengevat hebben wij aangetoond dat de bovengenoemde vier mutaties de functionele activiteit van MMP14 elk op een unieke manier verstoren, hetgeen restulteert in een vergelijkbaar klinisch fenotype.

Eén aspect van het WS en FTHS fenotype dat onze belangstelling heeft is acne [1, 6, 10, 11, 42]. Hoewel het alom bekend is dat acne gekenmerkt wordt door cysteuze talgklieren oftewel comedonen, is het onbekend waarom dergelijke cysteuze veranderingen optreden. De talgklier is in principe een holle, vertakte epitheliale structuur die

gescheiden is van de type I collageen-rijke dermis door een basaal membraan [43]. Vorming van dergelijke structuren hangt doorgaans af van *de novo* lumenformatie en vertakkende morfogenese [44]. Planaire celpolariteit (PCP) en ECM remodellering spelen een cruciale rol in deze twee processen en kunnen, indien verstoord, leiden tot cystevorming [45-48]. Opvallend genoeg zijn meerdere celtypen afhankelijk van de katalytische activiteit van MMP14 voor vertakkende morfogenese, en tevens van celmembraanlocalisatie van MMP14 voor PCP [49-52]. Daarom hypothetiseerden wij dat verstoorde luminogenese en vertakkende morfogenese van de talgklier door verminderde functie van MMP14 of SH3PXD2B (gezien hun hierboven beschreven directe interactive) ten grondslag kan liggen aan comedovorming. Aangezien acne in onze patiënten succesvol werd behandeld met 13-cis retinoïnezuur, hypothetiseerden wij dat dit medicament het veronderstelde defect in vertakkende morfogenese zou kunnen corrigeren [1]. In **Hoofdstuk 3** beschrijven wij een pilotstudie die het effect van *Mmp14* of *Sh3pxd2b* knockdown (KD) dan wel retinoïden op lumenvorming en vertakkende morfogenese van epitheelcellen *in vitro* onderzoekt [53, 54]. We toonden aan dat KD van deze genen lumenvorming in gereconstrueerd basaal membraan (Matrigel™) verstoorde en vertakkende morfogenese in type I collageengel verminderde, terwijl retinoïden beide processen direct stimuleerden. Verstoorde lumenvorming door KD van *Mmp14* of *Sh3pxd2b* impliceert dat PCP afwijkend is [52]. Verder suggereert de verkorte tubuluslengte in gecrosslinkte type I collageenmatrix dat KD van *Mmp14* of *Sh3pxd2b* de ECM degradatie verstoort [55, 56]. Tenslotte kan de KD de celdeling inhiberen, wat de kleinere organoïden in type I collageengel kan verklaren. De laatste twee mechanismen zijn waarschijnlijk gekoppeld, aangezien eerder is aangetoond dat IMCD cellen geïsoleerd uit *Mmp14* KO muizen *in vitro* een proliferatief defect hadden door verlies van de collagenolytische activiteit van MMP14 [57]. Het feit dat KD van *Mmp14* of *Sh3pxd2b* eenzelfde effect veroorzaakt *in vitro* suggereert dat een gemeenschappelijke functionele pathway aangedaan is. Vanwege haar rol in ECM remodellering, is podosoomfunctie de meest waarschijnlijke kandidaat. Retinoïden zouden hun effect kunnen uitoefenen door podosoomvorming te stimuleren, aangezien we in **Hoofdstuk 2** hebben aangetoond dat 13-cis retinoïnezuur het transport van MMP14 S466P naar podosomen *in vitro* stimuleerde. Dit kan het therapeutisch effect van 13-cis retinoïnezuur in de behandeling van acne verklaren, hoewel aanvullende weefselspecifieke effecten waarschijnlijk ook een rol spelen [58, 59]. Toekomstig onderzoek dat onze experimenten met sebocyten herhaalt, zal moeten uitwijzen of deze processen een rol kunnen spelen in de morfogenese van talgklieren. Een dergelijk 3D sebocytmodel kan mogelijk gebruikt worden in screening naar anti-acne farmaca.

Naast acne wordt het WS fenotype gekenmerkt door craniofaciale dysmorfie en gegeneraliseerde osteopenie [1, 6]. De meeste aangedane craniofaciale skeletelementen zijn afgeleid van de van neurale lijst (NL), de voornaamste embryonale structuur die bij-

draagt aan de ontwikkeling van het gelaat [60-62]. Na hun inductie, delamineren NL cellen en migreren ze uitvoerig, waarvoor ECM remodellering essentieel is [60, 61, 63-65]. Aangezien MMP14 betrokken is bij ECM remodellering en invasieve celmotiliteit, hypothetiseerden wij dat verstoorde NL migratie ten grondslag ligt aan de craniofaciale dysmorfie van WS [4, 5, 21, 66]. Om deze hypothese te testen en de onderliggende oorzaak van osteopenie bij WS te bestuderen, besloten wij een zebravismodel te ontwikkelen. Zebravissen vormen dezelfde skeletweefsels als mensen, die zich bovendien op een vergelijkbare wijze ontwikkelen. Dit geldt met name voor de schedelbeenderen van de zebravis, waarvan de meerderheid is afgeleid van de NL [67-70]. Zebravissen hebben twee goed geconserveerde MMP14 paralogen, Mmp14a en Mmp14b, die tijdens de ontwikkeling in het mesenchym van de kop tot expressie komen [71-76]. De snelle uitwendige ontwikkeling en optische transparantie van het zebravisembryo maken bovendien live *in vivo* beeldvorming mogelijk [77-79]. In **Hoofdstuk 4** hebben wij de CRISPR/Cas9 techniek gebruikt voor knockout (KO) van zowel *mmp14a* als *mmp14b* in zebravissen. Onze *mmp14a/b* KO vissen toonden essentiële aspecten van het WS fenotype, inclusief verminderde groei, geleidelijk verergerende craniofaciale afwijkingen, hyperkyfose, osteopenie (hoewel beperkt tot de schedel), en een verkorte levensduur [1, 6]. In tegenstelling tot onze hypothese, waren NL inductie, delaminatie, farynxbooginvasie, en differentiatie tot craniofaciaal kraakbeen onaangetast door *mmp14a/b* KO. Zoals eerder gesuggereerd, zijn voorheen beschreven craniofaciale defecten in *mmp14a* en *mmp14b* morfanten waarschijnlijk aspecifieke neveneffecten van de gebruikte Morfolino oligonucleotiden [74, 75, 80]. In onze *mmp14a/b* KO larven was de skeletmineralisatie tijdens metamorfose onaangetast [81]. Volwassen mutanten hadden een verstoorde enchondrale/perichondrale en intramembraneuze ossificatie van zowel NL- als mesoderm-afgeleide beenderen, wat verder tegen een NL-specifiek problem pleit [68, 82]. Aangedane botten bevatten in het algemeen relatief weinig botmatrix met een afwijkende collageeninhoud en clusters multinucleaire cellen, en een relatief volumineuze, ongeorganiseerde kraakbeenkern. In muizen verstoort verlies van MMP14 de tijdige verwijdering van kraakbeen tijdens beide vormen van ossificatie, wat gepaard gaat met overmatige resorptie van gemineraliseerde botmatrix. Dit resulteert in vergelijkbare botafwijkingen als aanwezig in onze *mmp14a/b* KO vissen [51, 83]. Hoewel *in vitro* is aangetoond dat MMP14 de osteoblastdifferentiatie stimuleert en osteoclastdifferentiatie en -activatie inhibeert, zal aanvullend onderzoek uit moeten wijzen welke processen en celtypes betrokken zijn in het skeletfenotype van onze *mmp14a/b* KO vissen [84-86]. Verder is het nog onbekend in welke mate afwijkende podosoomfunctie betrokken is in het WS fenotype. Analyse van zebravissen met verschillende combinaties van *mmp14a/b*, *sh3pxd2b* en/of *mmp2* KO kan helpen om de samenwerking van de respectievelijke eiwitproducten in botremodellering te begrijpen. Tenslotte kan de *mmp14a/b* KO vis gebruikt worden voor de ontwikkeling van medicatie voor botafwijkingen in WS alsook osteopenie in het algemeen.

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